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Simian Virus 40 Host Range/Helper Function Mutations Cause Multiple Defects in Viral Late Gene Expression†

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Simian virus 40 (SV40) deletion mutants *dl*A2459 and *dl*A2475 express T antigens that lack the normal carboxy terminus. These mutants are called host range/helper function (*hr/hf*) mutants because they form plaques at 37°C on BSC-1 and Vero monkey kidney cell lines but not on CV-1p monkey kidney cells. Wild-type SV40 can provide a helper function to permit growth of human adenoviruses in monkey kidney cells; the *hr/hf* mutants cannot. Progeny yields of *hr/hf* mutants are also cold sensitive in all cell lines tested. Patterns of viral macromolecular synthesis in three cell lines (Vero, BSC-1, and CV-1) at three temperatures (40, 37, and 32°C) were examined to determine the nature of the growth defect of *hr/hf* mutants. Mutant viral DNA replication was similar to that of the wild type in all three cell lines, indicating that the mutations affect late events in the viral lytic cycle. In mutant-infected Vero cells, in which viral yields were highest, late mRNA levels were similar to those observed during wild-type infection. Levels of viral late mRNA from mutant-infected CV-1 and BSC-1 cells at 32 and 37°C were reduced relative to those of wild-type-infected cells. The steady-state level of the major viral capsid protein, VP1, in mutant-infected CV-1 cells was reduced to the same extent as was late mRNA. The synthesis of agnoprotein could not be detected in mutant-infected CV-1 cells but was readily detected in CV-1 cells infected by wild-type SV40. Primer extension analyses indicated that most late mRNAs from mutant-infected CV-1 cells utilize start sites downstream from the major wild-type cap site (nucleotide 325) and the agnoprotein initiation codon (nucleotide 335). These results indicate that deletion of the carboxyl-terminal domain of T antigen affects viral late mRNA production, both quantitatively and qualitatively. The agnoprotein is detected late in the wild-type SV40 lytic cycle and is thought to play a role in the assembly or maturation of virions. Reduced *hr/hf* progeny yields could result from decreased capsid protein synthesis and, in the absence of detectable levels of agnoprotein, from inefficient use of available capsid proteins.

The simian virus 40 (SV40) large tumor (T) antigen is a 708-amino-acid polypeptide expressed throughout the infection cycle in permissive monkey kidney cells (for a review, see reference 54). This protein is required for replication of viral DNA (8, 34, 47, 49), autoregulation of SV40 early transcription (1, 52), and transactivation of the SV40 late promoter (6, 25, 26). T antigen is also essential for SV40 to immortalize and to transform nonpermissive rodent cells to a malignant phenotype (27, 50).

T antigen is modified posttranslationally by phosphorylation (51), glycosylation (22), acylation (32), ADP ribosylation (19), and adenylylation (5). T antigen occurs in monomeric, dimeric, and larger oligomeric forms and in complexes with cellular proteins, including DNA polymerase α (45), p53 (33), and the retinoblastoma susceptibility gene product, Rb (14). Different forms of T antigen may be involved in its various biochemical activities. These activities include ATPase (9, 18), helicase (46), and specific binding to the SV40 origin of DNA replication (23, 39, 53). Each of these activities has been mapped to specific regions of the T-antigen molecule (10, 13, 37). The viral DNA replication function requires that these three activities be present in the same T-antigen monomer (13, 55). Together, these domains encompass sequences extending from approximately amino acid 130 through 600 (13).

The extreme carboxyl terminus of large T antigen mediates a distinct function, the host range/adenovirus helper function (*hr/hf*). Most monkey cell lines infected by human adenoviruses produce very low yields of viral progeny. The block to productive adenovirus infection occurs at a late stage of the adenovirus life cycle and can be overcome by the carboxyl terminus of SV40 T antigen (17, 40). This can be provided either by coinfection with SV40 or by infection with adenovirus/SV40 hybrid viruses (for a review, see reference 29). These hybrid viruses encode either large T antigen, carboxyl-terminal fragments of T antigen, or adenovirus/SV40 fusion proteins containing the carboxyl terminus of large T. We have shown that the adenovirus helper function activity of T antigen is contained within the carboxyl-terminal 34 amino acids of T antigen (11, 38).

Previously, we described deletion mutants of SV40 (*dl*A2459, *dl*A2465, and *dl*A2475) which do not express the normal carboxyl terminus of large T (12, 13, 55). In addition to being defective for adenovirus helper function, these mutants are unable to form plaques on CV-1p monolayers at 37°C. This defect in plaque formation can be complemented by SV40 mutants producing a T antigen with a normal carboxyl terminus (55, 56) or by a mutant in which the carboxyl-terminal 26 amino acids of large T are fused to the amino-terminal 346 amino acids of VP1, the major capsid protein (57). This demonstrates that the *hr/hf* function is separable from the rest of T antigen.

The growth characteristics of the *hr/hf* mutants are cell line and temperature dependent (12). The mutants form plaques in CV-1p cells only at 40°C, in BSC-1 cells at 37 and 40°C, and in Vero cells at 32, 37, and 40°C. Interestingly, human adenoviruses also grow productively in Vero cells

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† This report is dedicated to George Khoury, whose many interests included the adenovirus helper function activity of SV40 large T antigen and whose curiosity and support we valued deeply.

(15) but not in CV-1 or other African green monkey kidney (AGMK) cell lines tested. The growth properties of *hr/hf* mutants indicate that the adenovirus helper function of T antigen is also required by SV40 for productive infection in CV-1 cells. Other *hr/hf* mutants have been described by Manos and Gluzman (35) and by Pipas (36).

In our initial characterization of mutant *dl*A2459 (56), we reported that the mutant genome replicates in CV-1 cells at 37°C and that a defect in late gene expression is responsible for the reduced mutant progeny production. In this report, we describe the patterns of viral late mRNA and VP1 synthesis during *dl*A2459 and *dl*A2475 infections of CV-1, BSC-1, and Vero cells at various temperatures. In Vero cells, *hr/hf* mutants produced approximately normal amounts of late mRNA, VP1, and viral progeny. In contrast, levels of late mRNA and VP1 were reduced in mutant-infected BSC-1 and CV-1 cells.

The agnoprotein, which is encoded in the leader region of some SV40 late mRNAs, is detected late in the SV40 lytic cycle (23) and is thought to be involved in the assembly and maturation of virions (3, 7, 42). We could not detect the agnoprotein after mutant infection of CV-1 cells. This agrees with results obtained by Khalili et al. (27), who investigated the properties of similar mutants. The major wild-type SV40 late mRNA start site is located upstream from the agnoprotein initiation codon (41, 54) and, therefore, most 16S late mRNAs contain the sequences necessary to synthesize the agnoprotein. In contrast, data presented here indicate that the start sites of most late mRNAs produced in *hr/hf* mutant-infected CV-1 cells mapped downstream of the agnoprotein initiation codon. There were no differences in the 16S late mRNA start sites used by wild-type SV40 and *hr/hf* mutants in Vero cells. The very low yield of progeny virions in mutant-infected CV-1 cells probably results from reduced production of late mRNAS and capsid proteins and, because of the absence of agnoprotein, from the inefficient use of capsid proteins.

MATERIALS AND METHODS

Cells, viruses, and plasmids. The African green monkey kidney cell lines CV-1, CV-1p, BSC-1, and Vero were maintained in Dulbecco modified minimal essential medium (DMEM) supplemented with 5 or 10% fetal calf serum–penicillin (10 U/ml)–streptomycin (10 μg/ml) in a humidified atmosphere containing 7% CO_2. CV-1p cells are a subline of CV-1 cells used for plaque assays. CV-1 cells were used for all other experiments.

The construction of mutant plasmids pdlA2459 and pdlA2475 has been described previously (12, 55). To prepare stocks of these mutants, Vero cells were transfected (57) with mutant viral DNA which had been separated from pBR322 sequences by digestion with *Eco*RI and recircularized by ligation at a low DNA concentration (5 μg/ml) with T4 DNA ligase. Stocks of SV40 wild type (WT830), a small-plaque strain (48), were prepared in Vero cells. Cells were infected with SV40 at a multiplicity of infection of 0.05. When most of the cells had rounded up but had not yet detached, they were scraped from the plate, suspended in a small volume of DMEM with 2% fetal calf serum, and sonicated. The cell debris was removed by centrifugation, and the viral stocks were stored at −80°C. Titers of wild-type and mutant stocks were determined by plaque assay on monolayers of BSC-1 cells, as previously described (56).

Ribonuclease protection analysis of late viral mRNA. Confluent cell cultures were infected with mutant or wild-type virus at a multiplicity of infection of 5 and were incubated at the temperatures indicated in the figure legends. Cytoplasmic RNA was prepared from cells harvested at 48 h (37 and 40°C cultures) or 96 h (32°C cultures) postinfection by the method of White et al. (58). SP6 bacteriophage RNA polymerase was obtained from Promega Biotec (Madison, Wis.) and used to prepare [^{32}P]UMP-labeled RNA probes for use in RNase protection mapping, following the directions provided by the enzyme supplier. RNA probes were synthesized from plasmid pSP64SVL (a generous gift from J. Alwine), which contained the SV40 genome opened at its *Bam*HI site and inserted downstream from the SP6 promoter in the orientation which permitted synthesis of RNA probes complementary to SV40 late mRNAs. The plasmid was linearized at the *Apa*I site (SV40 nucleotide 2258). After denaturation, cytoplasmic RNA was annealed to an excess of probe RNA overnight at 60°C, and hybrids were digested at 37°C with RNase A (40 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and T_1 (2 μg/ml; Sigma). Protected fragments were analyzed by electrophoresis on 6% polyacrylamide–7 M urea gels, followed by autoradiography.

Analysis of viral capsid protein. At various times after infection, cells were harvested in lysing buffer (0.15 M NaCl, 0.02 M Tris hydrochloride [pH 8.0], 1% Nonidet P-40) from cultures infected with *dl*A2475, *dl*A2459, or wild-type SV40. Lysates from equal numbers of infected cells were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose by electroblotting. Blots were incubated first with rabbit antiserum to SDS-disrupted SV40 virions, and then with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma). Immunodetection was with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium, as described by Blake et al. (4). The SV40 antiserum recognizes only VP1.

Analysis of agnoprotein. *dl*A2459-, *dl*A2475-, or wild-type-infected CV-1 cells were pulsed-labeled with [^{14}C]arginine (323.5 mCi/mmol; New England Nuclear-DuPont) in arginine-free DMEM for 1 h. Cells were lysed in SDS sample buffer (7 M urea, 7 mM sodium phosphate, monobasic, 1% SDS, 0.1% 2-mercaptoethanol, 0.01% bromophenol blue) and analyzed on a polyacrylamide gel containing 0.1 M sodium phosphate (pH 7.2)–0.1% SDS–6 M urea–15% polyacrylamide-bisacrylamide (30:0.8) (44).

Primer extension analysis. A 30-base-pair (bp) oligonucleotide primer complementary to late mRNA and extending from SV40 nucleotides 1498 to 1468 was synthesized and labeled by using [α-^{32}P]ATP and T4 polynucleotide kinase. Samples (10 μg) of cytoplasmic RNA extracted from mutant- and wild-type-infected cells were denatured with primer in 5 μl of hybridization buffer (80% formamide, 40 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] [PIPES] [pH 6.7], 0.4 M NaCl, 1 mM EDTA) at 85°C for 10 min and annealed at 60°C for 4 h. Reaction volumes were brought to 50 μl in 50 mM Tris (pH 8.5)–10 mM $MgCl_2$–40 mM KCl–1 mM dithiothreitol–250 μM of each deoxynucleotide triphosphate; 5 U of avian myelobastosis virus reverse transcriptase was added, and samples were incubated at 42°C for 1 h. RNA was hydrolyzed in 0.5 N NaOH; reaction mixtures were neutralized with 0.5 N HCl and ethanol precipitated, and cDNAs were analyzed by electrophoresis on 6% polyacrylamide–7 M urea gels.

RESULTS

The carboxyl terminus of SV40 large T antigen is a separate and separable functional domain which is required

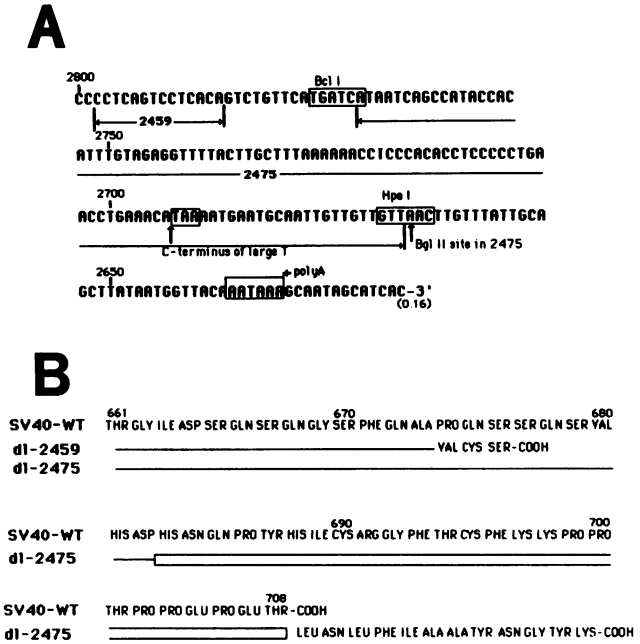

FIG. 1. (A) Nucleotide sequence of the 3′ end of the early region of the SV40 genome. Shown are the locations of the deletions in the *dl*A2459 and *dl*A2475 genomes, the termination codon for large T antigen, and the early mRNA polyadenylation signal. (B) Sequence of the carboxyl-terminal portion of the T antigens encoded by wild-type SV40, *dl*A2459, and *dl*A2475. The single line refers to residues in mutant proteins that are identical to the wild type. The boxed area represents residues that are absent from the *dl*A2475 T antigen.

for productive infection of most monkey kidney cell lines by SV40 (35, 36, 57) or human adenoviruses (16, 20). We constructed SV40 mutants *dl*A2459 (55) and *dl*A2475 (12) whose T antigens lack this domain and have shown that these mutants have a host range and cold-sensitive phenotype (12, 57). Figure 1 shows the structure of the 3′ end of the early region of the SV40 genome, the sequences deleted in the genomes of mutants *dl*A2459 and *dl*A2475, and the corresponding T-antigen amino acid sequences. *dl*A2459 has a 14-bp deletion of nucleotides 2798 to 2785 that causes a reading frame shift and the production of a 673-amino-acid T antigen lacking the carboxyl-terminal 35 amino acids of wild-type T antigen. *dl*A2475 is deleted from nucleotides 2770 through 2669 and produces a 682-amino-acid T antigen that lacks the 26 carboxyl-terminal amino acids. Both contain non-wild-type amino acids at their carboxyl termini. The growth characteristics of these two mutants are very similar, but the growth defect is slightly more severe for *dl*A2475 than for *dl*A2459. This could result from *dl*A2475 having 12 non-wild-type amino acids at the carboxyl terminus, while *dl*A2459 has only 3 non-wild-type amino acids (Fig. 1).

In our previous study (12), we reported that mutant progeny yields were decreased 75- to 80-fold in CV-1 cells at 37 and 40°C, 25- to 60-fold in BSC-1 cells, and about 10-fold in Vero cells, relative to those of the wild type. In all cell lines examined, mutant yields were reduced most severely at 32°C. We showed previously that *dl*A2459 DNA replicated normally in CV-1p cells at 37°C (57), and we concluded that the host range/helper function of large T antigen is required after the onset of viral DNA replication. We have since analyzed *dl*A2459 and *dl*A2475 DNA replication in Vero and BSC-1 cells, as well as CV-1 cells, at 37 and 32°C (data not shown). In all cases, we found that mutant viral DNA replicated nearly as efficiently as wild-type DNA. Other laboratories have analyzed similar carboxyl-terminal T-antigen mutants and reported them to be competent for viral DNA replication (35, 36). In an effort to understand the molecular basis of the host range and cold-sensitive defects of the *hr*/*hf* mutants, we compared the patterns of late viral macromolecular synthesis in various monkey kidney cell lines infected at different temperatures by wild-type SV40, *dl*A2475, or *dl*A2459.

Levels of late viral mRNA. We examined late viral mRNA levels in CV-1, BSC-1, and Vero cells infected with *dl*A2459, *dl*A2475, or wild-type SV40. Total cytoplasmic RNA was isolated from infected cells and hybridized to an RNA probe that protects the 3′ ends of all SV40 late mRNAs. After RNase digestion, the protected fragments were analyzed on denaturing polyacrylamide gels (Fig. 2). Figure 2A shows the results of an analysis performed on RNA from mutant and wild-type infections of the three cell lines incubated at 37°C. Equal quantities of viral late mRNA were detected in mutant- and wild-type-infected Vero (Fig. 2A, lanes 8, 9, and 10) and BSC-1 (Fig. 2A, lanes 5, 6, and 7) cells. However, decreased levels of mutant viral late mRNA were observed in mutant-infected CV-1 cells at 37°C (compare Fig. 2A, lanes 3 and 4, with Fig. 2A, lane 2). This indicates that the absence of the normal carboxyl terminus of T antigen correlated with decreased levels of SV40 late mRNA in CV-1 cells but not in Vero or BSC-1 cells.

Mutant viral yields are most cold sensitive in CV-1 cells. We compared viral late mRNA levels in mutant- and wild-type-infected CV-1 cells incubated at 32, 37, and 40°C (Fig. 2B). The levels of mutant viral late mRNAs were clearly affected by incubation temperature. Very little viral late mRNA from mutant-infected cells was detected at 32°C (Fig. 2B, lanes 2 and 3); slightly higher levels of late mRNAs were detected in mutant-infected cultures maintained at 40°C (Fig. 2B, lanes 9 and 10) than at 37°C (Fig. 2B, lanes 6 and 7). Interestingly, wild-type late mRNA production was slightly cold sensitive (compare Fig. 2B, lanes 1 and 4), which correlates with the observation that even wild-type SV40 is slightly cold sensitive in CV-1 cells (12).

Viral capsid protein synthesis. We compared the accumulation of VP1, the SV40 major capsid protein, in mutant- and wild-type-infected Vero and CV-1 cells (Fig. 3). These two cell lines are the most and least permissive, respectively, for growth of the *hr*/*hf* mutants. Lysates for Western blotting were harvested on three consecutive days after infection at 37°C (Fig. 3B and E) and 40°C (Fig. 3C and F) and on four consecutive days after infection at 32°C (Fig. 3A and D) to accommodate the slower time course of infection at 32°C. In Vero cells (Fig. 3D to F), VP1 accumulated to similar levels in wild-type- and mutant-infected cultures. The slightly lower levels of VP1 in *dl*A2475 infection versus *dl*A2459 (Fig. 3E) were observed reproducibly. In contrast, in CV-1 cells (Fig. 3A to C), VP1 accumulation during mutant infection was reduced significantly relative to that during wild-type infection. These reduced levels of capsid protein correspond to the observed levels of late mRNA. At 37°C in CV-1 cells, in which levels of mutant late mRNAs were reduced 5- to 10-fold (Fig. 2B), VP1 levels were reduced to a similar degree. The stability of VP1 was also examined by pulse-chase labeling of mutant- and wild-type-infected Vero and CV-1 cells incubated at different temperatures. VP1 had the same stability in mutant- and wild-type-infected cells (data not shown).

Synthesis of agnoprotein. A 61-amino-acid protein, the

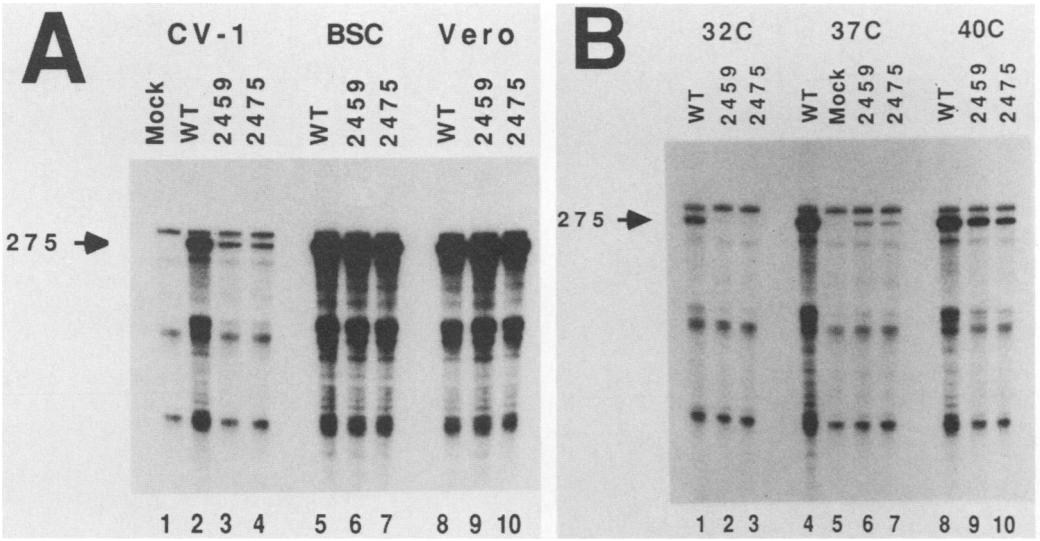

FIG. 2. RNase protection analysis of SV40 late mRNAs. Total cytoplasmic RNA was prepared from infected or mock-infected cultures and annealed with [^{32}P]-labeled antisense RNA probe. After hybridization, RNA was digested with RNase A and T_1 and the protected RNA hybrids were analyzed on denaturing 6% polyacrylamide-urea gels, as described in Materials and Methods. In hybrids between probe and SV40 late mRNAs, a 275-nucleotide band is protected (arrows). (A) Total cytoplasmic RNA was prepared for protection analysis from CV-1 (lanes 1 to 4), BSC-1 (lanes 5 to 7), or Vero (lanes 8 to 10) cells infected with wild-type SV40 (lanes 2, 5, and 8), *dl*A2459 (lanes 3, 6, and 9), or *dl*A2475 (lanes 4, 7, and 10), or the cells were mock infected (lane 1). (B) Total cytoplasmic RNA was prepared for protection analysis from CV-1 cells infected with wild-type SV40 (lanes 1, 4, and 8), *dl*A2459 (lanes 2, 6, and 9), *dl*A2475 (lanes 3, 7, and 10), or from mock-infected cells (lane 5). Incubation temperatures were 32°C (lanes 1 to 3), 37°C (lanes 4 to 7) or 40°C (lanes 8 to 10).

agnoprotein, is encoded in the leader region of the major class of SV40 16S late mRNAs (23). Carswell and Alwine (7) suggested a role for this protein in the efficient nuclear localization of VP1. A direct interaction between the agnoprotein and VP1 in the assembly of virions was suggested from studies of pseudorevertants of mutants that do not produce the agnoprotein (3). A role for the agnoprotein in the efficient release of virions from SV40-infected cells was proposed by Resnick and Shenk (42). Since progeny fields in CV-1 cells infected by *hr/hf* mutants were reduced to a greater extent (50- to 100-fold) than were the levels of VP1 (5- to 10-fold), we examined the production of agnoprotein in mutant-infected CV-1 cells.

The agnoprotein is rich in arginine and can be distinguished from other cellular and viral proteins by in vivo labeling with [^{14}C]arginine and fractionation of the small-molecular-weight proteins on polyacrylamide-urea gels (23, 44). Figure 4 shows the results of such an analysis. Although a [^{14}C]arginine-labeled protein of the appropriate size was readily detected 40 h after infection by wild-type SV40 (Fig. 4, lane 1), this protein could not be detected in mutant- or mock-infected CV-1 cells (Fig. 4, lanes 2 to 4), even after prolonged exposure of the autoradiogram (data not shown). This result confirms the finding that agnoprotein is not produced in CV-1 cells infected by similar SV40 *hr/hf* mutants (27) and suggests that the failure to produce agnoprotein in CV-1 cells is a general property of mutants of this class.

Analysis of viral late mRNA start sites. SV40 16S late mRNAs have multiple cap sites and therefore contain various amounts of leader sequence upstream from the VP1 coding region (41, 54). Those that start at or 5′ to the major cap site (nucleotide 325) and have the most common splicing pattern encode the agnoprotein (Fig. 5B). This is approximately 80% of 16S late mRNAs in wild-type-infected cells (41). 16S messages with other splicing patterns or those using cap sites downstream of the agnoprotein AUG (nucleotide 335) do not encode the agnoprotein. A failure to produce agnoprotein could reflect either defects in translation of 16S mRNAs encoding the agnoprotein or alterations in 16S late mRNA structures. To distinguish among these possibilities, we mapped the cap sites of 16S late mRNAs present in mutant- and wild-type-infected CV-1 and Vero cells by primer extension analysis. Autoradiograms of the reverse-transcribed products are shown in Fig. 5A. In Vero cells (Fig. 5A, lanes 5 to 7), identical patterns of primer extension were seen after mutant and wild-type infections. A major band corresponding to transcription initiation at nucleotide 325 is indicated.

In CV-1 cells, the band corresponding to transcription initiation at the major cap site (nucleotide 325) was the major band seen in wild-type-infected cells (Fig. 5A, lane 1) but was barely detectable in cells infected by *dl*A2459 (Fig. 5A, lane 2) or *dl*A2475 (Fig. 5A, lane 3). For both mutants, the major primer extension products detected corresponded to species of SV40 16S late mRNA that are present but relatively rare in wild-type-infected cells and that initiate downstream (nucleotides 465 and 482) of the agnoprotein AUG at nucleotide 335. Although start sites at nucleotides 465 and 482 were reported by Reddy et al. (41), they note that the site at nucleotide 465 is next to an AU sequence known to be adjacent to capped structures on SV40 late mRNAs (21), whereas the start site at nucleotide 482 is not. Our present data do not exclude the possibility that either or both of these extension products arose from premature termination of the cDNA. However, it is clear from our data that 16S late mRNAs able to encode the agnoprotein were very rare in mutant-infected CV-1 cells. We have repeated this experiment several times under different conditions; we have never observed a prominent band corresponding to the major start site at nucleotide 325 in analyses using multiple different samples of mutant late mRNA.

We conclude from this analysis that synthesis of the agnoprotein does not occur at detectable levels after *hr/hf*

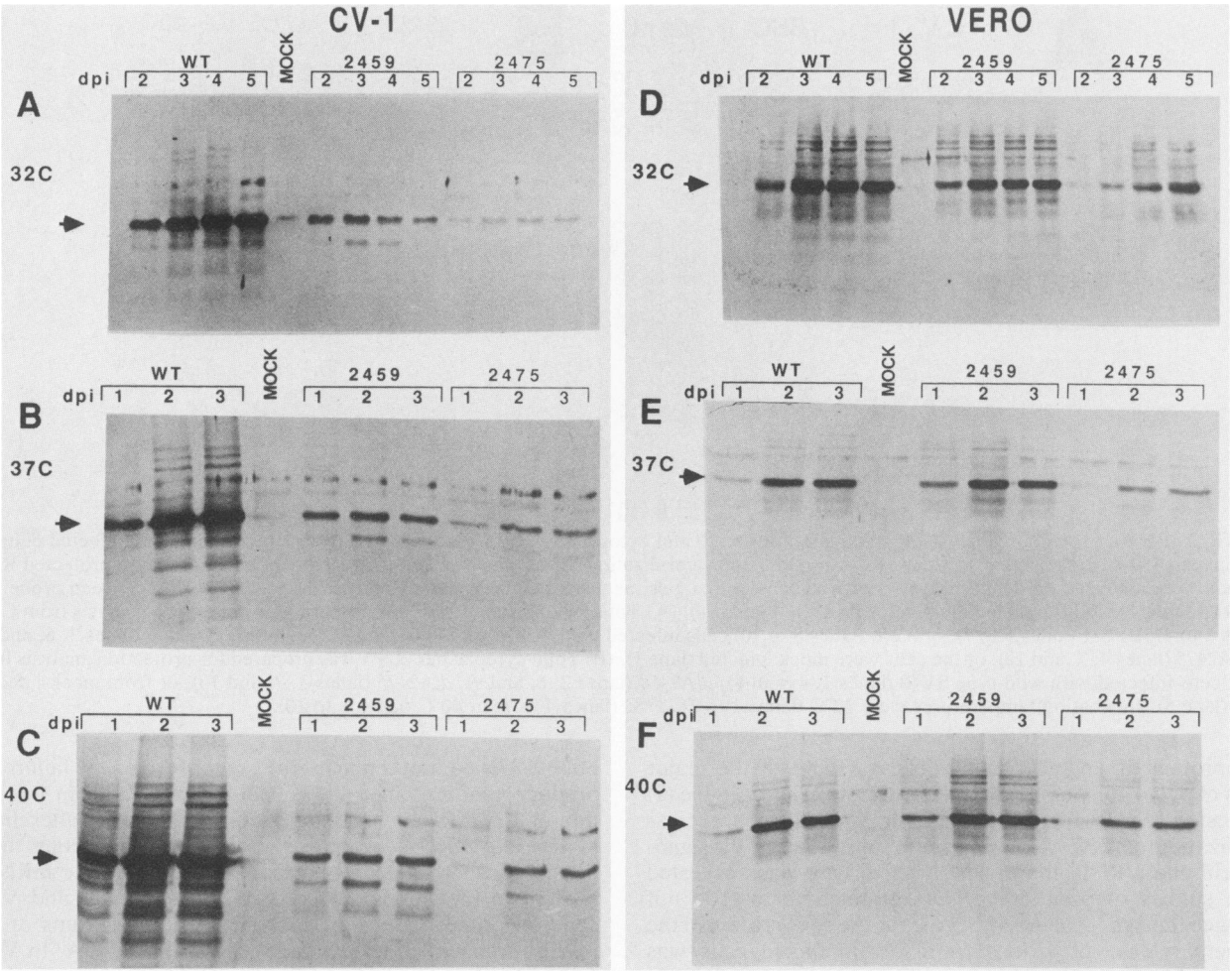

FIG. 3. Western immunoblotting analysis of SV40 capsid protein accumulation. Lysates from mutant-, wild-type-, or mock-infected CV-1 (panels A to C, respectively) and Vero cells (panels D to F, respectively) that had been cultured at 32°C (panels A and D), 37°C (panels B and E), or 40°C (panels C and F) were electrophoresed on 8% SDS-polyacrylamide gels. After transfer to nitrocellulose, capsid protein was detected by treatment first with rabbit antiserum to SDS-disrupted SV40 virions, which recognizes only VP1, and then with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. Immunodetection was as described in Materials and Methods. Numbers above the lanes indicate the days postinfection (dpi) that lysates were prepared.

mutant infection of CV-1 cells because the 16S late mRNAs produced lack the coding information for this protein. Both the steady-state levels and the structures of the SV40 late mRNAs were altered in *hr/hf* mutant-infected CV-1 cells.

DISCUSSION

SV40 deletion mutants which produce large T antigens that lack the normal carboxyl terminus show host range and cold-sensitive behavior for plaque formation and progeny yield and are also absolutely defective for adenovirus helper function (12). They are referred to as *hr/hf* mutants.

In this study, we analyzed the levels of viral late mRNA and viral late proteins after mutant and wild-type SV40 infection of three AGMK cell lines. Of the lines tested, CV-1 cells are the most restrictive and Vero cells are the most permissive for *hr/hf* mutant growth. BSC-1 cells are intermediate for mutant growth. Since the mutants show cold sensitivity, some experiments were conducted at 32, 37, and 40°C. Our results can be summarized as follows.

(i) Viral late mRNA accumulated to similar levels in Vero and BSC-1 cells infected by *hr/hf* mutants and wild-type SV40 but not in CV-1 cells (Fig. 2A). In CV-1 cells at 37°C, the mutants accumulated only 10 to 20% as much late viral mRNA as wild type. Even less late message was present at 32°C in mutant-infected CV-1 cells. The levels of mutant late message were somewhat greater in CV-1 cells incubated at 40°C.

(ii) The major capsid protein VP1 accumulated to nearly equal levels in Vero cells infected with wild-type SV40 or *hr/hf* mutants (Fig. 3). However, mutant-infected CV-1 cells failed to accumulate VP1 to wild-type levels. The extent of the decrease in the amount of VP1 in mutant-infected CV-1 cells reflected the decreased level of late mRNA. Therefore, the *hr/hf* mutation appears to affect late mRNA accumulation but not its translation. Whether there is a defect in transactivation, in transcription elongation, or at some post-transcriptional step in mRNA metabolism will require additional studies.

(iii) Synthesis of the agnoprotein was detected in wild-type-infected CV-1 cells but not in *hr/hf*-infected CV-1 cells (Fig. 4). Primer extension analysis showed that the agnoprotein could not be produced at detectable levels during mutant

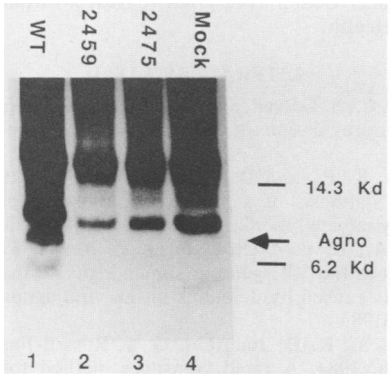

FIG. 4. Analysis of the synthesis of the agnoprotein. Plates (35 mm diameter) of confluent CV-1 cells were infected with wild-type SV40 (lane 1), *dl*A2459 (lane 2), or *dl*A2475 (lane 3), or they were mock infected (lane 4). At 40 h postinfection, cells were labeled with 5 μCi of [^{14}C]arginine per plate for 1 h in DMEM lacking arginine plus 2% dialyzed fetal calf serum. Lysates were harvested and analyzed on an SDS–15% polyacrylamide gel containing 6 M urea, as described in Materials and Methods. Positions of ^{14}C-labeled molecular size markers and the agnoprotein (Agno) are indicated.

infection of CV-1 cells because of altered patterns of mutant late mRNA start sites. Although the mutants and wild type showed identical patterns of mRNA cap sites in Vero cells, almost all of the 5′ ends of mutant viral 16S mRNAs in CV-1 cells mapped downstream of the agnoprotein initiation codon (Fig. 5). The major sites used in mutant-infected CV-1 cells are sites which are used to form minor species of 16S late mRNA during wild-type infection (41, 54).

The results of our primer extension analyses do not agree with conclusions reached by Khalili et al. (27), who reported no differences in late mRNA leader structures in CV-1p cells infected with wild-type SV40 and *hr/hf* mutants similar to our mutants. The primer used in their studies (nucleotides 460 to 520) would not have detected late mRNAs initiated at nucleotides 465 and 482, which we found to be the major species of 16S late mRNA in mutant-infected CV-1 cells. We also examined the cap sites of 19S late mRNAs in mutant- and wild-type-infected CV-1 and Vero cells. In Vero cells, mutant and wild-type late 19S mRNAs use the same cap sites with the same frequencies; in CV-1 cells, most mutant 19S late mRNA start sites map downstream from the major wild-type site (T. Stacy and C. N. Cole, unpublished data). Although the conclusions reached by Khalili et al. (27) do not agree with ours, the data in their report clearly show decreased use of the major cap site at nucleotide 325 in 19S late mRNA isolated from mutant-infected CV-1p cells.

Vero cells are most permissive for *hr/hf* mutant infection; wild-type amounts of viral DNA, late mRNA, and VP1 were produced. Previous studies suggested that mutant viral progeny yields were reduced up to 10-fold in Vero cells relative to those of the wild type. We now believe that the actual virion yields in mutant-infected Vero cells are higher than yields calculated from standard plaque assays. These plaque assays are performed with BSC-1 cells because monolayers of Vero cells do not survive long enough for use in these assays. Approximately 15% of mutant-infected BSC-1 cells produce sufficient virions to form plaques, whereas plaques are formed from nearly 100% of wild-type-infected cells (T. Stacy and C. N. Cole, manuscript in preparation).

Although the two deletion mutants, *dl*A2459 and *dl*A2475, behaved similarly with respect to host range and helper function properties, they are not identical. *dl*A2459, with a

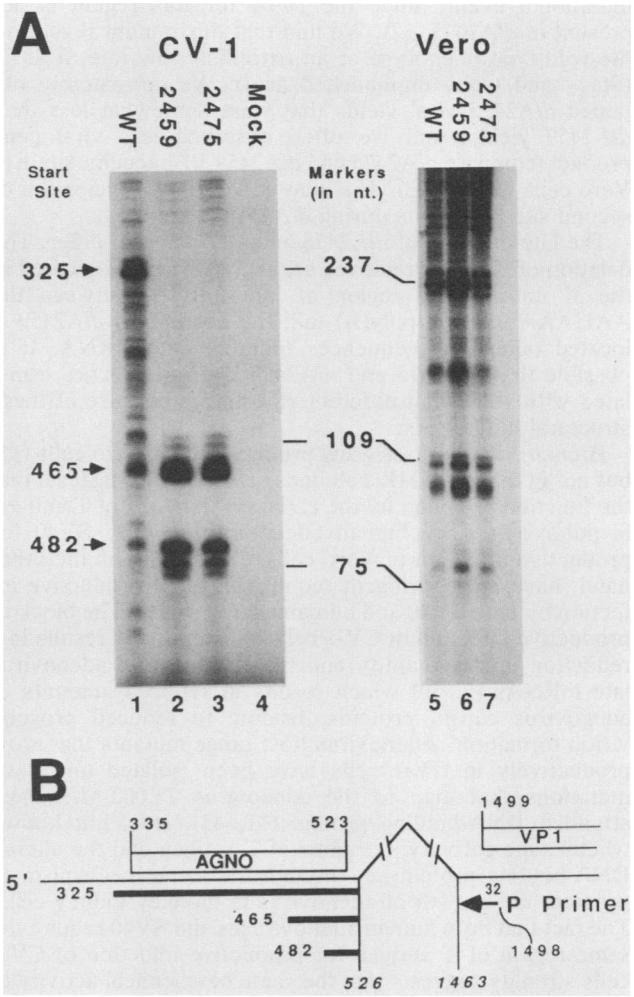

FIG. 5. Primer extension analysis of SV40 late 16S mRNA 5′ ends. (A) CV-1 and Vero cells were infected with wild-type SV40 (lanes 1 and 5), *dl*A2459 (lanes 2 and 6), or *dl*A2475 (lanes 3 and 7), or they were mock infected (lane 4). Cytoplasmic RNA was harvested at 48 h postinfection, and primer extension reactions were performed as described in Materials and Methods. ^{32}P-labeled cDNA products were analyzed on 6% polyacrylamide–7 M urea gels. The SV40 nucleotide positions of 16S mRNA start sites (41, 55) are indicated by arrows; the positions of nucleic acid size markers are also shown. (B) The SV40 late leader region and splice junctions for 16S mRNAs are shown. The translation initiation and termination codons for the agnoprotein and the initiation codon for VP1 are shown above the line. The major 16S mRNA cap site at nucleotide position 325, as well as minor cap sites at positions 465 and 482 are indicated below the line. The splice donor and acceptor sites are in italics. The ^{32}P-labeled 30-base oligonucleotide primer extends from SV40 nucleotides 1498 to 1468.

14-bp frame-shift deletion upstream of the region encoding the carboxyl terminus of T antigen, can revert to a wild-type phenotype with the deletion of only 1 more bp in the vicinity of the original deletion, thus restoring the wild-type T-antigen reading frame. We have determined the proportion of revertants in the progeny of a single growth cycle of *dl*A2459 to be approximately 1% (Stacy and Cole, unpublished results). The proportion depends on the timing of the initial reversion mutation(s) and may also involve cellular factors. In contrast, the wild-type T-antigen carboxyl terminus cannot be restored to *dl*A2475 T antigen by a single

mutational event, since the DNA for this region is not present in *dl*A2475 (12). We find that this mutant reverts to the wild-type phenotype at an extremely low rate, if at all (Stacy and Cole, unpublished data). We consistently obtained *dl*A2475 viral yields that were somewhat less than *dl*A2459 yields, and we often observed less viral gene product (compare *dl*A2475 and *dl*A2459 VP1 accumulation in Vero cells in Fig. 3E). This may reflect the participation of second-site revertants during *dl*A2459 infection.

The late mRNAs of *dl*A2475 and *dl*A2459 also differ. The deletion of *dl*A2475 removes sequences normally located in the 3′ untranslated region of late mRNA, between the AAUAAA and the poly(A) tail; the deletion in *dl*A2459 is located outside of sequences found in late mRNA. It is possible that *dl*A2475 and *dl*A2459 late mRNAs are translated with somewhat different efficiencies because of these structural differences.

Human adenoviruses grow productively in Vero cells (15) but not in other AGMK cell lines. This finding suggests that the function provided by the carboxyl terminus of T antigen is not required by human adenoviruses or by SV40 for productive infection of Vero cells. CV-1 cells, on the other hand, have more stringent requirements for productive infection by both SV40 and human adenoviruses. The block to productive infection of CV-1 cells by adenovirus results in a reduction in the quantity and translatability of adenovirus late mRNAs (2, 30) which results in reduced amounts of adenovirus capsid proteins, leading to reduced progeny virion formation. Adenovirus host range mutants that grow productively in CV-1 cells have been isolated and have mutations that map to the adenovirus 72,000-M_r single-stranded DNA-binding protein (31, 43). It is not known whether the carboxyl terminus of T antigen and the altered DNA-binding protein act through the same mechanism to promote the growth of adenovirus in monkey kidney cells. The fact that both human adenoviruses and SV40 require the same region of T antigen for productive infection of CV-1 cells strongly suggests that the same biochemical activity of T antigen is important for human adenovirus and *hr/hf* mutant growth.

The *hr/hf* mutant phenotype is complex. Mutants produce viral DNA in CV-1 cells but fail to accumulate wild-type levels of late mRNAs or the capsid protein VP1. These mutants also fail to produce the agnoprotein in CV-1 cells. The mutants will form plaques on CV-1 cells that express the agnoprotein constitutively; however, agnoprotein supplied in *trans* does not restore the mutant progeny yields to wild-type levels (Stacy and Cole, in preparation). This finding suggests that providing the agnoprotein permits efficient use of available capsid proteins but cannot restore late mRNA to wild-type levels. The primary defect caused by *hr/hf* mutations is still not known. The data presented here indicate that these mutations result in decreased levels of late mRNA and that the late mRNAs have an altered distribution of cap sites. This could reflect a direct effect of T antigen on late transcription or an indirect effect due to alterations in the nature of the progeny DNA molecules which serve as templates for late transcription.

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